

Mycorrhizal Status of Obligate and Facultative Epiphytic Ferns in a Valdivian Temperate Forest of Patagonia, Argentina

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ABSTRACT.—Arbuscular mycorrhizae (AM) influence the growth, morphology and fitness of the plant species they colonize. Despite the abundance and importance of epiphytes in forest dynamics, little is known about AM in these plants. Abundant epiphytes are present in the Valdivian temperate forests of South America, where ferns are one of the most important components of the epiphytic vascular flora. The aim of this work was to analyze the occurrence of AM in sporophytes of obligate and facultative epiphytic fern species in a Valdivian temperate forest of Patagonia, Argentina. We examined the roots of 10 epiphytic fern species. Roots of 66 sporophytes were collected from tree trunks and branches between 0.5 and 2 m height above the ground and were stained by using a modified Phillips and Hayman (1970) method. Of the 10 species examined in the present study two were found to be obligate epiphytes, six were facultative epiphytes and two could not be classified because few individuals were found. Most of them lacked AM structures, except for seven *Hymenophyllum* sporophytes which were rooted in soil and possessed scarce knobby hyphae and structures similar to coils and vesicles. Abundant dark septate fungi were observed within the cortex of all samples. The absence of AM in epiphytes may be explained by different ecological or taxonomic reasons, but in this study we suggest that root morphology may be an important factor influencing mycorrhizal behavior in ferns.

KEY WORDS.—epiphytes, ferns, arbuscular mycorrhizae (AM), dark septate fungi (DSF), Patagonia

Autotrophic epiphytes are plants that grow upon other plants which only provide physical support and are not parasitized. Plants restricted to epiphytic habitats are called “obligate epiphytes” and those that occur both epiphytically and terrestrially are known as “facultative epiphytes” (Benzing, 1990; Janos, 1993). Vascular epiphytes constitute approximately 10% of all vascular flora (Kress, 1986) and play an important role in forest dynamics by contributing to species richness and by trapping and storing atmospheric nutrients, making them temporally unavailable to terrestrial plants (Benzing, 1990).

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Mycorrhizae generally influence plant fitness by improving nutrient uptake and increasing plant resistance to drought and other environmental stress factors (Smith and Read, 1997; Read, 1999). Despite the importance and abundance of epiphytes, little is known about mycorrhizal associations in these plants (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993; Michelsen, 1993). Epiphytic habitats are usually considered extreme environments for plants because they often are subjected to great variation in temperature and in water and nutrient availability (Benzing, 1990). Therefore, it is generally expected that mycorrhizae may be an important adaptation for epiphytes (Benzing, 1990; Lesica and Antibus, 1990; Janos, 1993; Rains *et al.*, 2003). Nevertheless, it has been observed that epiphytic species are usually non-mycorrhizal (Allen *et al.*, 1993), facultative mycorrhizal (commonly mycorrhizal when they grow terrestrially and inconsistently mycorrhizal when they grow epiphytically) (Maffia *et al.*, 1993; Michelsen, 1993; Nadarajah and Nawawi, 1993), and that when they have AM, colonization is usually low (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Rowe and Pringle, 2005).

Studies concerning the occurrence of mycorrhizae in epiphytes are few and only the Orchidaceae and Ericaceae families have been relatively well studied (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Dearnaley, 2007). Some species included in the Bromeliaceae (Allen *et al.*, 1993; Rabatin *et al.*, 1993; Rowe and Pringle, 2005) and other predominant families of vascular epiphytes (such as the Araceae, Cyclanthaceae and Piperaceae) have also been analyzed (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Janos, 1993; Maffia *et al.*, 1993; Michelsen, 1993). However, the mycorrhizal status of epiphytic species included in families other than those mentioned here remains an open question. Therefore, much more information is needed before generalizations about mycorrhizae in epiphytic environments can be made (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993).

Abundant epiphytes are present among the vegetation of the Valdivian temperate forests of South America. Within these forests, seedless vascular plants, mostly ferns (monilophytes, see Pryer *et al.*, 2004 and Smith *et al.*, 2006), are one of the most important components of the epiphytic vascular flora. The mycorrhizal status of this group of plants is poorly understood (Dhillion, 1993; Zhao, 2000) and much less is known about this symbiosis in epiphytic species.

This research was conducted in Puerto Blest, where previous studies have demonstrated the presence of AM in different plant species (Chaia *et al.*, 2006), including *Equisetum bogotense* HBK and *Lycopodium paniculatum* Desv. ex Poir., two species of seedless vascular plants which were found to be facultative mycorrhizal (Fernández *et al.*, 2008). The aim of the present study was to analyze the occurrence of AM in sporophytes of different obligate and facultative epiphytic fern species.

MATERIALS AND METHODS

Study area.—The Valdivian temperate forests of South America are included in the Subantarctic Province (Subantarctic Domain, Antarctic Region). This



FIG. 1. Geographical location of the study area. **a:** Nahuel Huapi National Park, Puerto Blest area is pointed out in a black rectangle, **b:** Puerto Blest region.

ecoregion is located in the Southern region of Argentina and Chile, from 35° to 48° S latitude between the Eastern slope of the Andes and the Pacific Ocean. Puerto Blest ($41^{\circ} 02' S$, $71^{\circ} 49' W$) is part of the Valdivian temperate forest region of Argentina and it is situated within the Nahuel Huapi National Park in Río Negro Province, Patagonia (Cabrera, 1976; Armesto *et al.*, 1995) (Fig. 1). It is one of the rainiest places in the country, with an average annual rainfall of 3000 mm and an annual average temperature of $9^{\circ} C$. The dominant tree species in this hydrophilic forest is *Nothofagus dombeyi* (Mirb.) Oerst. In some areas, this species forms mixed forests with *Fitzroya cupressoides* (Molina) IM Johnst., *Saxegothaea conspicua* Lindl., *Dasyphyllum diacanthoides* (Less.) Cabrera. and *Laureliopsis philippiana* (Looser) Schodde (Dimitri, 1972; Brion *et al.*, 1988; Correa, 1998). Trunks and branches of these tree species are usually covered by different epiphytes, including lichens, mosses and several ferns. Epiphytic ferns present in Puerto Blest are small plants that range in size from 2 to 20 cm.

Sampling.—Five samplings were conducted in Puerto Blest between spring 2004 and autumn 2006. Sporophytes of epiphytic ferns were sampled by random walk from trunks and branches of different tree species, such as *N. dombeyi*, *S. conspicua*, *D. diacanthoides* and *L. philippiana*, which were situated at least 5 m away from the footpath. The epiphytic specimens were collected from 0.5 to 2 m height above the ground. Species that were found to be also growing on the forest soil were considered as facultative epiphytes and root samples were collected from epiphytic as well as from the conspecific

terrestrial specimens. One individual of *Hymenophyllum dentatum*, a species that is reported to be epiphytic (Rodríguez, 1995) but was found only on the ground, was also sampled.

Sporophytes were carefully removed from the substrate in order to preserve most of the root system and were stored in labeled plastic bags and refrigerated at 4°C until processed. Roots from all samples were separated from the rest of the plant, carefully washed and cleaned under a stereoscopic microscope (Olympus SZ 30) so as to remove all root pieces belonging to other plants. Samples were fixed in 70% formalin-acetic acid-alcohol (FAA).

Analyses of the samples.—All samples were stained using a modified Phillips and Hayman (1970) method (see Fernández *et al.*, 2008). For each individual three slides were made, each of them with ten stained root pieces of approximately 1 cm length mounted in glycerol. Root pieces were examined with a light microscope (Olympus BX40) at 20× magnification. The criterion used in this study for the determination of AM was the presence of arbuscules in at least one individual of each species. Structures corresponding to AM or other root fungi were documented as brightfield images, which were captured with a digital camera (Sony ExwaveHAD) and Image-Pro Plus 4.1.0.0. analysis software for Windows.

RESULTS

A total of 66 sporophytes belonging to ten species in three families were sampled. Ten specimens (15%) corresponded to *Asplenium dareoides*, eight (12%) to *Grammitis magellanica* and 48 (73%) to various species of the family Hymenophyllaceae. In Puerto Blest, *A. dareoides* and *G. magellanica* are the only representatives of the Aspleniaceae and Polypodiaceae families respectively (Brion *et al.*, 1988; de la Sota *et al.*, 1998). At least five sporophytes were found for most of the species, except for *H. cuneatum*, *H. falklandicum* and *H. ferrugineum*, which were represented by only one or two specimens (Table 1).

Most of the samples (73%) were collected from epiphytic habitats and we observed that they frequently had soil particles attached to the outside of the roots, probably as a consequence of their proximity to the ground. Terrestrial sporophytes (27%) were collected from loose organic soils with abundant leaf and wood litter, 2 (11%) of them belonged to *A. dareoides* and 16 (89%) to five species of *Hymenophyllum* (Table 1). All the sporophytes examined presented extensive, thin (less than 2 mm wide) and fibrous root systems with abundant root hairs. Baylis (1975) and St. John (1980) described this root system morphology as “graminoid”.

Most of the sporophytes lacked AM structures, but in seven terrestrial samples (11% of the total specimens analyzed), corresponding to *H. pectinatum* and *H. seselifolium* (Table 1), scarce knobby hyphae and structures similar to coils and vesicles were observed (less than 5% of the root length examined) (Fig. 2). However, arbuscules were not detected in any of these specimens, so it is questionable if these plants can be designated as mycorrhizal.

TABLE 1. Occurrence of arbuscular mycorrhizae in epiphytic and conspecific terrestrial sporophytes of ferns of Puerto Blest. **AM/Total**: number of individuals in which we observed AM / total of individuals examined, **(n°)**: number of samples in which we observed structures similar to hyphal coils and vesicles, so it is doubtful whether these plants can be designated as mycorrhizal.

TAXON	LIFE FORM		
	Epiphytic	Terrestrial	AM/Total
Aspleniaceae			
<i>Asplenium dareoides</i> Desv.	8	2	0 / 10
Polypodiaceae			
<i>Grammitis magellanica</i> Desv.	8	0	0 / 8
Hymenophyllaceae			
<i>Hymenophyllum cuneatum</i> Kunze	0	1	0 / 1
<i>Hymenophyllum falklandicum</i> Baker	1	0	0 / 1
<i>Hymenophyllum ferrugineum</i> Colla	2	0	0 / 2
<i>Hymenophyllum pectinatum</i> Cav.	10	5	(4) / 15
<i>Hymenophyllum seselifolium</i> C Presl	4	7	(3) / 11
<i>Hymenophyllum tortuosum</i> Hook. & Grev.	5	2	0 / 7
<i>Hymenophyllum umbratile</i> Diem & JS. Licht.	4	1	0 / 5
<i>Serpillopsis caespitosa</i> (Gaudich.) C Chr.	6	0	0 / 6

Abundant extra- and intracellular hyphae of regularly septate and generally melanized fungi frequently associated with microsclerotia and “cerebriform structures” were present in all root systems (Fig. 3). They corresponded to dematiaceous root endophytes usually named dark septate fungi (DSF).

DISCUSSION

Of the ten species examined in the present study, *G. magellanica* and *S. caespitosa* were found to be obligate epiphytes (only growing as epiphytes), while *A. dareoides* and five species of *Hymenophyllum* (*H. cuneatum*, *H. pectinatum*, *H. seselifolium*, *H. tortuosum* and *H. umbratile*) were facultative epiphytes (growing either epiphytically or terrestrially). These observations agree with de la Sota *et al.* (1998), who described the same habits for these species. We considered *H. cuneatum* as facultative epiphytic even though only one terrestrial sporophyte was collected because it is described as epiphytic in the literature (Rodríguez, 1995). *Hymenophyllum falklandicum* and *H. ferrugineum* could not be classified as obligate or facultative epiphytes because only one or two individuals per species were found (Table 1).

Representatives of the Aspleniaceae, Polypodiaceae and most of the Hymenophyllaceae families in Puerto Blest clearly lack AM colonization. Chaia *et al.* (2006) and Fernández *et al.* (2008) observed that different plant species growing in areas adjacent to our study site had AM. Furthermore, Chaia *et al.* (2006) found out that in Puerto Blest the AM soil infective capacity ranges from 0.6 to 1.7 IU g/soil. These findings indirectly demonstrate that

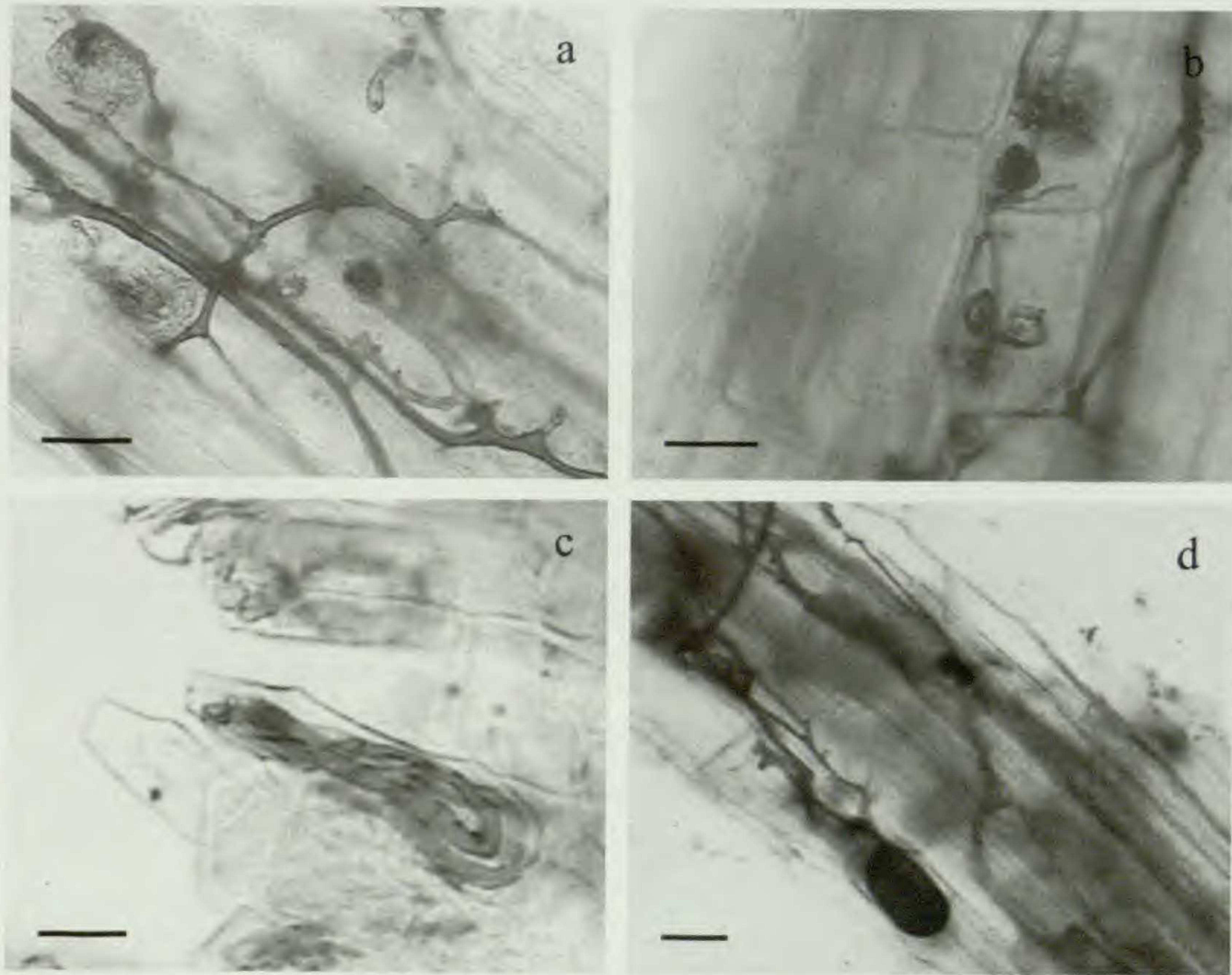


FIG. 2. Fungal colonization similar to arbuscular mycorrhizas in *H. pectinatum* (a,b) and *H. seselifolium* (c,d). **a**: Extra and intracellular mycelia where some septa can be observed, **b,c**: Structures resembling intracellular coils, **d**: Intracellular hyphae and a vesicle-like structure. Scale bars: **a,b**=20 μ m; **c,d** = 50 μ m.

there is AM inoculum in the soil where the samples were collected. However, terrestrial specimens of *A. dareoides*, *H. cuneatum*, *H. tortuosum* and *H. umbratile* did not form AM despite the presence of inoculum in the soil, which supports the non-mycorrhizal behavior of these species. Even though we found structures similar to vesicles and coils in roots of four out of five terrestrial sporophytes of *H. pectinatum* and in three out of seven terrestrial sporophytes of *H. seselifolium* (Fig. 2), it was not possible to record them as mycorrhizal because arbuscules (the diagnostic feature of the symbiosis) were absent and the colonization percentage was very low (less than 5%). Additionally, it is known that some other fungi are capable of forming structures similar to those of AM within roots of some epiphytic plants (Brundrett, 2002; Rowe and Pringle, 2005). These structures can also stain dark blue (Fig. 2c,d) and in consequence they may be mistaken for AM (Janos, 1993). The presence of such fungi could explain the presence of this type of structures in roots of *H. pectinatum* and *H. seselifolium*.

The absence of mycorrhizae in the epiphytic fern species examined in this work is concordant with previous studies that have analyzed this symbiosis in other species belonging to the same families (Cooper, 1977; Gemma *et al.*, 1992; Zhao, 2000; Wang and Qiu, 2006), but differs from some others that have

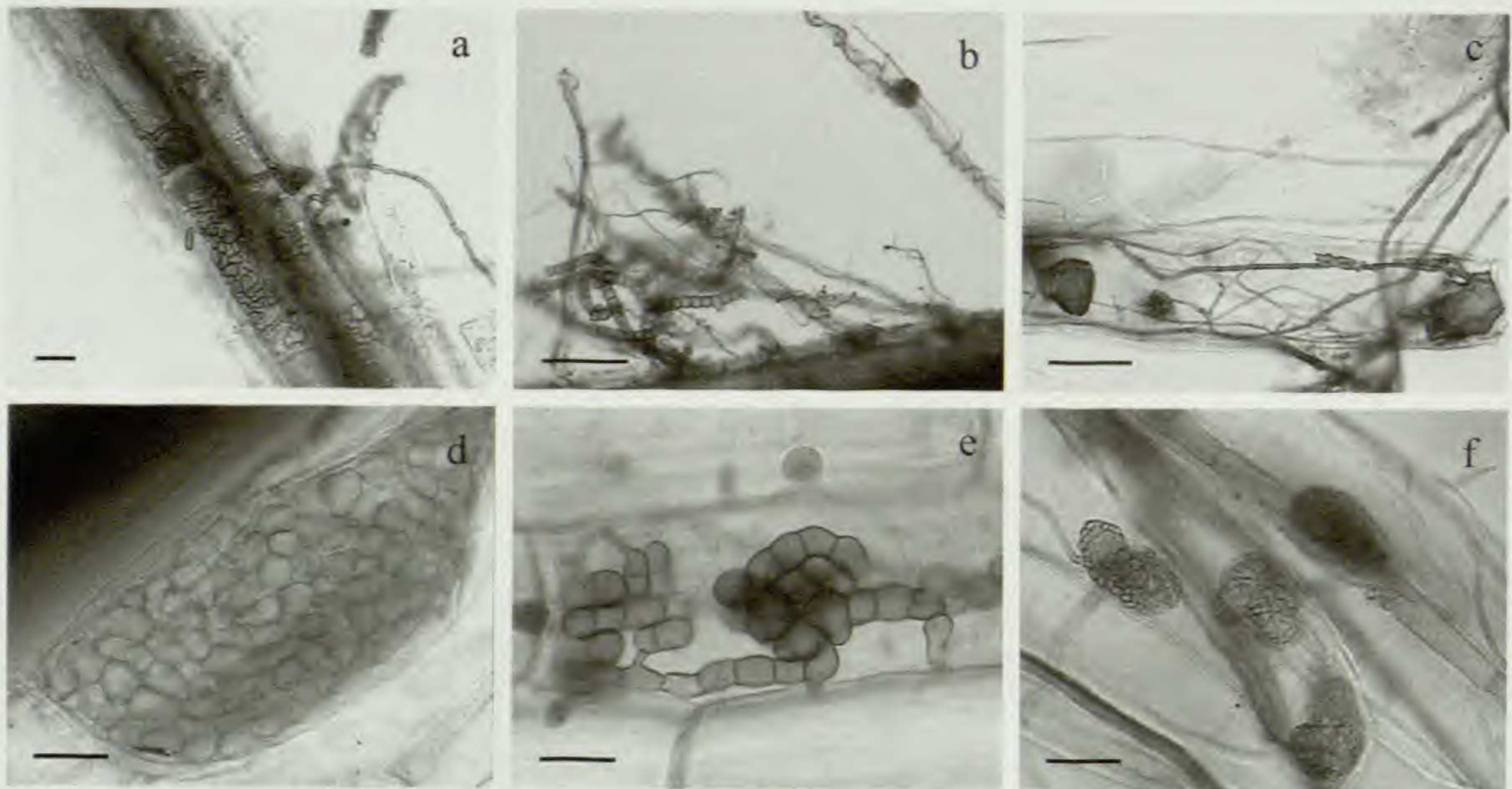


FIG. 3. Dark septate fungi in roots of epiphytic ferns of Puerto Blest. **a:** Extraradical hypha and intracellular microsclerotia in *S. caespitosa*, **b:** General view of extraradical DSF colonization in *G. magellanica*, **c:** Intracellular septate hyphae and associated vesicle-like structures in *H. ferrugineum* (structures stained blue; color not shown) **d:** Moniliform hyphae (structures stained blue; color not shown) within cortical cells of *A. dareoides*, **e:** Highly melanized moniliform hyphae in roots of *H. tortuosum*, **f:** Characteristic cerebriform microsclerotia in *H. seselifolium*. Scale bars: **a**=50 μm ; **b,c**=100 μm ; **d,e,f**=20 μm .

reported the occurrence of AM in the same group of plants (Cooper, 1976; Iqbal *et al.*, 1981; Gemma *et al.*, 1992; Muthukumar and Udaiyan, 2000; Zhao, 2000; Wang and Qiu, 2006). Our results are also consistent with Lesica and Antibus (1990) and Michelsen (1993), who examined 13 and eight species of epiphytic ferns in Costa Rica and Ethiopia respectively (including five species of *Asplenium* and one of *Hymenophyllum*), and described them as non-mycorrhizal. Nadarajah and Nawawi (1993) studied the mycorrhizal status of 19 fern species in Malaysia. They observed that all these species were non-mycorrhizal in epiphytic habitats and that eight of them were able to form AM vesicles and/or coils when they were rooted in soil. Nevertheless, as it occurred in this work, these authors did not detect arbuscules in any of the samples.

Although it has been suggested by several authors that AM are generally absent (Allen *et al.*, 1993) or rare (Bermudes and Benzing, 1989; Lesica and Antibus, 1990; Maffia *et al.*, 1993, 2000; Michelsen, 1993; Nadarajah and Nawawi, 1993) in epiphytes, Rains *et al.* (2003) found abundant mycorrhizal epiphytes in a Costa Rican cloud forest. This apparent discrepancy suggests that the occurrence, and possibly the role, of mycorrhizae in epiphytic habitats is variable and may be related to taxonomic affinities and diverse ecological factors (Tester *et al.*, 1987; Lesica and Antibus, 1990; Michelsen, 1993).

Different explanations have been suggested for the paucity of AM in epiphytic habitats. For example, it might be that these environments are too dry and exposed to support the fungus or that the rate of photosynthesis

(which is relatively low in epiphytes, see Benzing, 1986) may be insufficient to maintain the symbiosis (Lesica and Antibus, 1990). Nutritional insufficiency, and consequently the inability to sustain the energy cost of mycorrhizae, has also been suggested as a major factor determining mycorrhizal success in epiphytes (Benzing, 1990; Janos, 1993). Some substrate analyses have demonstrated that trunks and canopy mats might be nutrient rich (Bermudes and Benzing, 1989; Lesica and Antibus, 1990), and it is known that high fertility suppresses AM of facultatively mycotrophic species (Janos, 2007). Another widespread explanation is that epiphytic habitats are deficient in AM inocula, so epiphytes usually do not have the opportunity of being colonized by mycorrhizal fungi (Lesica and Antibus, 1990; Janos, 1993; Michelsen, 1993). This suggestion contrasts with several authors (McIlveen and Cole, 1976; Rabatin *et al.*, 1993; Janos *et al.*, 1995; Mangan and Adler, 2000) who have reported that AM spores and mycelia may be dispersed to epiphytic environments by several vectors (e.g., mainly birds, ants and small mammals). Moreover, other studies have established that many epiphytes lack AM despite the presence of spores and external mycelia in the substrate (Lesica and Antibus, 1990; Maffia *et al.*, 1993; Rabatin *et al.*, 1993).

In this study it was possible to compare the mycorrhizal status of six facultative epiphytic fern species. In the case of *A. dareoides*, *H. cuneatum*, *H. tortuosum* and *H. umbratile* all specimens lacked AM, and in most of the terrestrial sporophytes of *H. pectinatum* (73%) and *H. seselifolium* (73%) fungal structures similar to those of AM were completely absent. Therefore, deficiencies in AM inocula or in nutrient acquisition (which are not limiting factors when plants are rooted in soil, as has been demonstrated in previous studies carried out in Puerto Blest) would not explain the non-mycorrhizal behavior observed in these plants. Thus, it is necessary to seek other explanations for this phenomenon. Another factor that might explain the absence of AM in epiphytic ferns of Puerto Blest is root system morphology. Plants with long root length (Koide and Li, 1991; Ryser and Lambers, 1995), small root diameter (Reinhardt and Miller, 1990; Manjunath and Habte, 1991; Hetrick *et al.*, 1992) and abundant long root hairs (Baylis, 1975; St. John, 1980; Schweiger *et al.*, 1995), like those observed in this study ("graminoid roots"), tend to be non-mycorrhizal (Baylis, 1975; St. John, 1980; Hetrick, 1991; Michelsen, 1993; Zangaro *et al.*, 2005).

Dark septate fungi (DSF) are conidial or sterile fungi that have been reported in several plant species from different habitats (Jumpponen and Trappe, 1998; Jumpponen, 2001). Characteristic inter- and intracellular hyphae and micro-sclerotia corresponding to DSF were observed in every specimen analyzed in this work (Fig. 3). This is in accordance with previous studies that have reported the existence of this type of fungi within the roots of angiosperm epiphytes (Bermudes and Benzing, 1989; Allen *et al.*, 1993; Janos, 1993; Rains *et al.*, 1993) and several fern species (Cooper, 1976; Dhillon, 1993; Fernández *et al.*, 2008). The presence of "cerebriform microsclerotia" (Fig. 2f) has not been described in the literature before, except in *E. bogotense* and *L. paniculatum*, two facultative mycorrhizal species also studied in Puerto Blest

(Fernández *et al.*, 2008). The presence of DSF within the roots of different fern species demonstrates that these plants are capable of forming plant-fungal associations with a diversity of fungal lineages (Winther and Friedman, 2007). According to Allen *et al.* (1993) and Rains *et al.* (2003), the lack of AM in most of the epiphytes in addition to the occurrence of DSF in many of them is notable and may suggest that DSF may function as “mycorrhizal fungi”, but further study is needed to test this hypothesis.

This study provides novel information about the occurrence of mycorrhizae in epiphytic ferns and it the first work in which the mycorrhizal status of epiphytic species of Patagonia, Argentina is recorded. More work on a larger scale is needed not only for understanding the behavior of epiphytic species of Puerto Blest but also for developing patterns of mycorrhizal activity in epiphytic habitats and for determining ecological explanations for the paucity of AM in these plants (Lesica and Antibus, 1990; Allen *et al.*, 1993; Nadarajah and Nawawi, 1993).

ACKNOWLEDGMENTS

We are grateful to Parques Nacionales for giving us the opportunity to work in a protected area and we thank Dr. Davis Janos for critically reviewing our manuscript and for his valuable comments on this work. Funds for this research were provided by grant PICT 22200 (FONCyT).

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